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For the invention entitled:

**METHOD FOR DIAGNOSING MULTIPLE
SCLEROSIS AND AN ASSAY THEREFORE**

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1 METHOD FOR DIAGNOSING MULTIPLE SCLEROSIS AND AN ASSAY

2 THEREFORE

3
4 FIELD OF THE INVENTION

5 The present invention relates to a method for the
6 detection of biological materials relating to the prediction,
7 diagnosis, or monitoring of progression of an autoimmune
8 disease, utilizing an assay system developed to measure levels
9 of biochemical markers involved in autoimmune disease. More
10 specifically, this invention relates to an assay for detecting
11 myelin basic protein (MBP) autoantibodies alone, and
12 alternatively in conjunction with the measurement of other
13 biochemical markers associated with multiple sclerosis (MS) and
14 related diseases. Most specifically, the present invention is
15 directed toward a process for initially diagnosing MS, and
16 toward the para-clinical work-up and routine monitoring of MS
17 patients as to disease progression.

18
19 BACKGROUND OF THE INVENTION

20 Multiple Sclerosis (MS) is a chronic inflammatory
21 demyelinating disease of the human central nervous system
22 (CNS). Pathologically, the disease presents focal areas of
23 myelin destruction, known as plaques or lesions (Vollmer,
24 1999). Myelin is present as a multilamellar sheath formed by

membranous extensions of oligodendrocytes within the CNS (Peters, 1960a; b). Its ability to insulate axons and facilitate rapid nerve conduction is attested to by its high lipid content (70% of the total weight). With the focal deterioration of the insulating myelin sheath, the lesions result in decreased conduction velocity through naked regions of the axons affected. Such primary demyelination manifests as the symptoms of MS. Such symptoms include motor weakness in one or more limbs, optic neuritis, diplopia, parasthesia, fatigue, and eventually paralysis and early morbidity (Pender, 1995). Although the aetiology is unknown, geographic, genetic, and immune factors, acting coordinately, often determine disease onset and severity. Given the early onset of the disease (with diagnosis in children increasing in frequency), usually in the third or fourth decade of life, MS is one of the leading causes of neurological impairment in young adults. The precise mechanism by which demyelinating lesions are formed in MS is not known, however the most promising theory is that demyelination occurs as a result of aberrant immunology resulting in immune attack of myelin. The association between histocompatibility phenotype and susceptibility in MS has immunological implications. Furthermore, clinical features in MS, such as the presumed long latent period, the chronic nature of the disease and the pattern of acute attacks followed by

remission are suggestive of an immunologically mediated disease. Pathologically, MS lesions are marked by an infiltration of numerous immune cells, especially T-cell lymphocytes and macrophages (Pender, 1995). Histological and immunological similarities between MS and the classic animal model of the disease, experimental allergic encephalomyelitis (EAE), have suggested that the disease may be T-cell mediated (Esiri, 1991). However, evidence of humoral immunity in MS is also abundant as reflected by the presence of B-cells and plasma cells within MS lesions and the elevated levels of cerebrospinal fluid (CSF) immunoglobulin G (IgG) in MS patients. Although the autoantigen responsible for MS has not been conclusively identified, myelin basic protein (MBP) has been proposed as a candidate autoantigen.

MBP is a cationic membrane-associated protein found in myelin in the CNS, in which it accounts for approximately 35% of the total myelin protein. In humans, there are four main isoforms generated by alternative splicing of a single transcript: 21.5 kDa, 18.5 kDa, 17.2 kDa and the 14 kDa isoforms (Kamholz, et al., 1986; 1988). The 18.5 kDa isoform, in which exon 2 is spliced out, is 170 amino acids in length (Carnegie, 1971) and is the most prominent isoform in mature human myelin (Moscarello, 1997; Schmidt, 1999). After the identification of MBP as the antigen associated with EAE,

1 antibodies against MBP were detected in the CSF of MS patients
2 and the antibody levels correlated with active MS disease
3 status.

4 In addition to MBP, protein markers worthy of
5 investigation regarding MS autoantigens are:

6 1. Proteolipid protein (PLP) is a 30 kDa hydrophobic
7 protein which constitutes approximately 50% of the total myelin
8 protein. PLP is expressed in CNS myelin and is found in the
9 myelin membrane (Lees and Brostoff, 1984).

10 2. S-100B (bb homodimer) is a 21 kDa acidic calcium
11 binding protein expressed primarily in astrocytes,
12 oligodendrocytes (Kimura et al., 1986; Richter-Landsberg and
13 Heinrich, 1995) and Schwann cells, as well as other cells and
14 tissues including adipose tissue, skeletal muscle, the retina,
15 salivary glands, and immune cells (Zimmer et al, 1995).

16 3. The expression of neuron specific enolase (NSE) in
17 oligodendroglial cells is associated with the period during
18 which myelination takes place. NSE has been proposed as a
19 biochemical marker for both neuronal tumors (glioblastoma,
20 astrocytoma, and Schwannoma) as well as small cell lung
21 carcinoma (Jorgensen, 1999), and NSE can be reliably detected
22 in reactive astrocytes after brain injury (Lin et al., 1994).
23 Although NSE has not been suggested as a marker in MS, it
24 deserves serious consideration.

4. Thrombomodulin (Tm) is a 75 kDa endothelial cell surface transmembrane glycoprotein which functions to indirectly activate protein C, by binding to thrombin and altering its procoagulant activity (Dittman and Majerus, 1990). Thrombomodulin is thought to be released into the circulation when the endothelial cells are damaged (Tsukada et al, 1995). Therefore, damage to endothelial cells precedes thrombomodulin release into the circulation. Presumably, damage to the endothelial cells forming the blood-brain barrier (BBB) releases Tm into the CSF and is subsequently transported into the peripheral blood. Therefore, Tm may be released prior to the onset of clinical symptoms of relapse.

Traditionally, a good biochemical marker for any disease is characterized by a high clinical sensitivity and specificity for only the disease of interest. In the case of MS, numerous biochemical markers, including MBP, NSE, S-100B, and PLP autoantibodies, MBP, and thrombomodulin have been examined. Although these possible diagnostic measures have shown some extent of non-specificity for MS and variable clinical sensitivities, ranging from 0-50%, were observed, these biochemical markers have shown promise as diagnostic and prognostic tools attested by the fact that clinical sensitivities generally increased in patients experiencing active disease. This observation is especially characteristic

1 of MBP autoantibody measurement in the CSF of MS patients.

2 Specific clinical signs and the temporal pattern of their
3 presentation in the individual MS patient indicate the clinical
4 course of the disease. Each disease course is remarkably
5 different from the other, although the precise definition of
6 the forms of the disease remains a constant source of debate
7 and undergoes continuous revision. To summarize, the majority
8 of cases of MS initially run a relapsing and remitting course
9 (Matthews, 1991). This form of the disease is characterized
10 by alternating phases of exacerbation/relapse (acute attack of
11 neurological deficit) of symptoms and subsequent recovery with
12 little or no residual deficits (Stinissen et al, 1997; Thompson
13 et al, 1997). The duration of relapse ranges from a period of
14 hours to months before any remission of the symptoms is
15 observed. In many cases, patients first experience some degree
16 of optic neuritis. Improvement/recovery after the first attack
17 is sufficient to identify remitting disease (Vollmer, 1999).

18 On average, the proportion of patients with relapsing-
19 remitting MS (RRMS) is 85% (Vollmer, 1999). RRMS presents with
20 a characteristic alternation of phases between an acute attack
21 of neurological deficit (relapse) and a period of recovery
22 (stable). Patients older than 40 usually have the progressive
23 form (Vollmer, 1999) of the disease. Secondary-progressive MS
24 (SPMS) is characterized by significant neurological deficits

1 that increase over time with relapses (Thompson et al, 1997).
2 Many patients with RRMS progress to SPMS on average ten to
3 fifteen years after the initial symptoms present, although some
4 older patients (over 40 years at onset) experience the
5 secondary progressive disease course from the outset.

6 Primary-progressive MS (PPMS) is less common and is
7 characterized by patients with a slow and steady progression of
8 impairments from onset of presentation without distinct attacks
9 (relapses) (Al-Omaishi et al, 1999). Rarely, a more malignant
10 form of MS occurs and is called the Marburg's variant.
11 Marburg's disease is characterized by an acute fulminant
12 monophasic course resulting in death three weeks to six months
13 after the onset of initial symptoms (Lassman et al., 1981).
14 Conversely, some MS patients can remain asymptomatic for 15
15 years or longer and are considered to follow a "benign" disease
16 course. Disease course is variable between individual MS
17 patients resulting in a continuum of severity which reflects an
18 equally variable pathology and possibly aetiology.

19 Studies on the pathology of the demyelinating lesions in
20 MS have led to the identification of numerous key players in
21 the multifactorial pathogenesis of the disease. The release of
22 components of the myelin sheath, evidence of blood-brain
23 barrier (BBB) damage, astrocytic proliferation early in the
24 formation of the plaque, gliosis, as well as infiltration of

1 macrophages and B-cells within the lesion, and CD8⁺ T-cells
2 surrounding the lesions are consistent features in MS
3 pathology. These pathological characteristics facilitate both
4 the identification of contributory factors in MS and provide
5 clues as to which biochemical markers might offer the most
6 utility in the diagnosis and prognostic monitoring of MS.

7 Although the CNS is usually an immune privileged site, the
8 BBB is damaged during acute inflammation in the CNS, as serum
9 proteins have been found throughout the lesions (Brosnan and
10 Raine, 1996), resulting in novel access of lymphocytes to
11 myelin and myelin components. While MBP autoantibody testing
12 may be more useful in diagnosing MS, the measurement of MBP
13 itself may indicate activity of lesions which would possibly
14 precede clinical symptoms of relapse and may suggest
15 immunodominant epitopes if the MBP material can be identified.

16 The diagnosis of MS presently relies heavily on clinical
17 examination and neuroimaging techniques. Due to the
18 heterogenous nature of the disease, accurate and rapid
19 diagnosis is not only made difficult but is very costly and
20 often quite painful, especially when the differential diagnosis
21 includes diseases such as Behcet's disease, sarcoidosis with
22 CNS involvement and monophasic demyelinating disease, including
23 optic neuritis and acute disseminated encephalomyelitis (ADEM).
24 ADEM is especially important in the differential diagnosis of

MS in children.

Although a clinical sensitivity of ~90% can be achieved using MBP autoantibody levels in the CSF, this method is unsuitable as a diagnostic tool for several practical reasons. CSF is extracted through lumbar puncture (LP) which is an invasive technique. Besides being uncomfortable to the patient, complications associated with LPs include prolonged headaches post-procedure, a risk of infection, and lengthy time duration for both patient and physician since a typical lumbar puncture often requires the patient's presence for several hours. Furthermore, the performance of LPs are reserved for experienced neurologists.

What has, until now, been lacking in the art, is a simple assay in which MBP autoantibodies are measured in the blood or in a blood product of MS patients or suspected MS patients. Unfortunately, no useful method currently exists for MS patients to monitor their disease status. Venipuncture has much less associated complications than LP, is usually performed as a standard procedure in any initial diagnostic work-up, and may be performed by individuals without any neurological background. In addition, the complications surrounding acid dissociation, as is required at present, would not be necessary. Since blood sampling can be performed frequently, MBP autoantibodies may demonstrate prognostic utility and

1 predict disease progression. What is therefore needed is a
2 reliable immunoassay which can be easily commercialized, could
3 be integrated into the diagnostic work-up in MS, and may
4 facilitate the monitoring of patients, including those involved
5 in novel MS therapeutic trials.

6 With the recommendation for earlier treatment in MS,
7 earlier and reliable diagnostic measures are of paramount
8 importance. Since a disease course featuring spontaneous
9 remissions make any treatment regimen problematic, tailoring
10 treatments to individual sub-classifications of disease course,
11 i.e., relapsing-remitting, primary-progressive, secondary-
12 progressive, represent a more effective treatment method.
13 Though the average duration of life is 10 to 20 years following
14 onset, in actuality, many patients live longer. Some patients
15 have frequent attacks and are rapidly incapacitated, while
16 others have remissions that last as long as 25 years. Drugs
17 such as interferon beta-1b (IFN β -1b) are more effective in RRMS
18 than SPMS. At present, clinical stratification of a patient is
19 generally achieved through the patient's history. Only in PPMS
20 (there is more spinal cord lesion involvement) is MRI utilized.
21 Inclusion criteria for almost all drug trials include specified
22 disease course.

23 Thus, if a minimally invasive technique involving at least
24 one reliable biochemical marker indicative of disease activity,

1 as well as disease course, could be employed not only for the
2 diagnosis of MS and the prognostic monitoring of the disease
3 for clinical trial, but also as a means of determining disease
4 sub-classification for patient study inclusion, a long felt
5 need would be satisfied.

6
7 DESCRIPTION OF THE PRIOR ART

8 In a detailed and extensive study for determining the
9 specificity of MBP autoantibody response in the CSF, Warren and
10 Catz (Eur. Neurol., 42 (1999), 95-104) determined that 98% of
11 other non-MS controls did not exhibit elevated anti-MBP
12 antibodies. Warren and Catz further reported that 90-95% of MS
13 patients with active disease had elevated levels of MBP
14 autoantibodies in the CSF. Despite the work conducted using
15 the CSF, the presence of MBP autoantibodies in the systemic
16 circulation has not been conclusively determined. Previous
17 work has favored measurement in the CSF and numerous
18 investigators have reported limited success with their
19 detection in serum. Experiments using serum samples have been
20 plagued by problems associated with high background
21 interference using a variety of assay methods. This is likely
22 the result of insufficient optimization of assays for the
23 enhanced sensitivity required for MBP autoantibody detection at
24 low titers in a matrix containing so many other proteins.

1 Furthermore, the lack of consistent methodology between
2 investigators may account for the apparent discrepancy between
3 their collective results. To illustrate this point, Reindl and
4 colleagues (Brain, 122 (1999), 2047-2056) investigated the
5 detection of anti-myelin/oligodendrocyte glycoprotein (anti-
6 MOG) and anti-MBP IgG in the sera and CSF of MS patients.
7 Using a Western blotting technique, positive detection of anti-
8 MBP IgG was achieved in 28% of the total MS population studied.
9 Since the nature of MBP autoantibodies in serum is of
10 relatively low titer, at an equivalent polyclonal dilution,
11 especially compounded with the issue of high background as seen
12 through the Westerns performed, it is now understandable that
13 patients could not be detected as positive.

14 Several investigators have established that autoantibodies
15 against several myelin and non-myelin proteins can be detected
16 at various frequencies in the CSF of MS patients. At the
17 forefront of this work is the detection of MBP autoantibodies
18 in the CSF of MS patients. While Panitch (Arch. of Neurol., 37
19 (1980), 206-209) reports a clinical sensitivity of 81% in his
20 MS patients, Warren and Catz (Annals of Neurol., 20, 1 (1986),
21 20-25) observed a sensitivity of 55% when free (no acid
22 dissociation) levels were assayed and a clinical sensitivity of
23 56% when bound fractions were measured.

24 Previous work performed in the CSF demonstrated that MBP

1 was a reliable indicator of relapse, correlated well with the
2 number of gadolinium-enhanced lesions on MRI, EDSS scores,
3 intrathecal IgM synthesis (Lamers et al, 1998), and levels of
4 CSF MBP correlated well with CSF free anti-MBP levels (Warren
5 and Catz, 1987).

6 The most consistent examination of MBP autoantibodies in
7 the CSF has been performed by Warren and Catz (1986-present).
8 Their extensive work rests on the supposition that MBP
9 autoantibodies are frequently complexed with MBP and in order
10 to detect the autoantibodies, acid dissociation with 1N acetic
11 acid for release is required. It should be noted that the
12 diagnostic utility of MBP autoantibodies rests in this domain
13 and that their diagnostic success lies in studying patients
14 defined as having active disease (Warren and Catz, 1986), that
15 is patients in relapse. Throughout their work, Warren and Catz
16 have utilized acid dissociation of CSF samples to break MBP-
17 autoantibody complexes, and their results are reported in sub-
18 classified groups of CSF autoantibody responses. These
19 responses are free (non-dissociated, % bound radioactivity),
20 total (dissociated), bound (total minus free) and a final
21 free/bound ratio. All patients experiencing acute
22 exacerbations demonstrated elevated levels of free MBP
23 autoantibodies. Patients in the progressive form of the
24 disease had elevated bound levels of anti-MBP. Elevated levels

1 of either free or bound anti-MBP were not detected in patients
2 with clinically inactive disease. This group reasoned that
3 long-term and/or repeated MBP release into the systemic
4 circulation is likely to enhance the immune memory for anti-MBP
5 synthesis. In further support of the increased frequency of
6 anti-MBP responses, patients with optic neuritis (ON) were
7 examined for their autoantibody response to MBP. Warren and
8 Catz found that ON patients demonstrated elevated levels of
9 free MBP autoantibodies by radioimmunoassay (RIA) (Warren and
10 Catz, 1994). It appears that anti-MBP is associated with
11 active phases of disease in MS especially since MS patients
12 frequently present initially with attacks of ON. However,
13 given the requirement of acid dissociation before testing, this
14 RIA procedure would not provide a simple method of testing.

15 Although the precise mechanism by which MBP is released
16 from the myelin membrane has not been entirely worked out,
17 measurements by several groups have revealed that MBP in the
18 CSF, when measured by RIA, can be a reliable indicator of
19 disease activity (Whitaker, 1977; Whitaker and Herman, 1988).
20 Patients exhibited the highest levels of CSF MBP if they were
21 experiencing acute exacerbation/relapse (Whitaker, 1977;
22 Thompson et al, 1985; Warren et al, 1985; Frequin et al, 1992
23 and Ohta et al, 2000), whereas the majority of clinically
24 stable/remitting patients had levels of CSF MBP comparable to

that observed in other neurological disease controls. Furthermore, in patients in clinical remission who had significantly elevated MBP levels, sub-clinical demyelination was also detected by MRI (Thompson et al, 1985) which supports the role of MBP in CSF as a marker for myelin destruction. CSF MBP levels were reduced to normal levels when previously relapsing patients were treated with high doses of methylprednisolone (Frequin et al., 1992). This reduction in CSF MBP correlated well with a decrease in intrathecal IgM synthesis, which is also known to increase during relapse. Taken together, MBP, having also significantly correlated with EDSS score during relapse and the number of gadolinium-enhanced lesions on MRI, has shown promise as an indicator of MS pathology (Thompson et al., 1985; Frequin et al, 1992). Unfortunately, the clinical utility of MBP in the serum of MS patients has not been pursued because the extent of MBP peptide generation by circulating proteases has not been determined, resulting in the lack of a sensitive assay which specifically measures circulating MBP/MBP-peptides. Measurement of MBP in serum would perhaps provide a simple means of monitoring the progression of disease in an individual patient. Little research has been reported for the clinical specificity of the MBP autoantibody response in serum, presumably because many investigators have had such limited success in their detection

1 in MS patients.

2 The Schumacher criteria for the diagnosis of MS is based
3 solely on clinical examination and history (a more detailed
4 discussion to follow). With the advent of neuroimaging
5 techniques in the 1970's and advances in electrophysiology in
6 the 1980's, demyelinating lesions suggestive of MS became
7 visually apparent in some cases even prior to their clinical
8 manifestations. In addition, abnormalities in CSF
9 immunoreactivity, such as the presence of oligoclonal bands,
10 increased IgG synthesis, and elevated IgG, became useful
11 diagnostic criteria, but none were definitive. The existing
12 clinical criteria did not accommodate the new laboratory
13 techniques for diagnosing MS. In 1983, Poser and his
14 colleagues published a new set of diagnostic guidelines which
15 added the demonstration of para-clinical lesions (i.e. lesions
16 visualized by MRI or CT scan or evoked-potential testing) as
17 part of the clinically definite MS diagnostic criteria, as well
18 as a new diagnostic category of laboratory-supported definite
19 MS which required the detection of oligoclonal bands in the CSF
20 or increased CNS synthesis of IgG, as measured by the CSF IgG
21 index (CSF IgG Index is determined by the ratio CSF/serum IgG:
22 CSF/serum albumin) (Link, 1991).

23 For prognostic purposes, unambiguous diagnostic criteria
24 for MS is of extreme importance for the patient. At the

1 present time, the definitive diagnosis of MS is often times a
2 lengthy process which renders the patient anxiously uncertain
3 for days or months regarding the diagnosis and prognosis. In
4 addition, defining a population of patients with "probable or
5 possible" MS aids in the prospective evaluation of novel
6 diagnostic tests for the disease (Poser et al., 1983) is often
7 problematic. The clinical trials for testing novel MS
8 therapies are now multi-centered and require a minimization of
9 the subjectivity involved in the clinically-based diagnostic
10 work-up performed by the examining neurologist. Resolution of
11 these issues could be achieved if a simple, rapid, and
12 clinically sensitive, diagnostic test was available to both
13 family physicians and neurologists alike.

14 U.S. Pat. No. 5,747,274 to Jackowski discloses a variety
15 of assay types, as well as various assay formats and automated
16 analyzer apparatus.

17 U.S. Patent No. 5,645,997 issued to Kline et al. describes
18 an assay for detecting antigens associated with multiple
19 sclerosis utilizing hybridomas to produce monoclonal antibodies
20 specific for MS-associated antigens. Peripheral blood
21 lymphocytes (PBL) are isolated from a blood sample, and then
22 processed to extract the MS-associated mitogens from the whole
23 PBLs, if they are present. Kline et al. also teaches the
24 administration of an effective dose of MS-associated antigens

1 or a fraction thereof or its associated nucleotide sequences or
2 a fraction thereof.

3 U.S. Patent No. 5,998,150 issued to Whitaker et al.
4 provides a method for determining the status of an MS patient
5 participating in an IFN β -1b trial, by measuring levels of
6 urinary myelin basic protein-like material in the patient for
7 predicting failure of remission or presence of a progressive
8 phase in MS. The measurement by radioimmunoassay, preferably
9 a double-antibody radioimmunoassay, is said to correlate with
10 the number of lesions or total area of lesions in an MS
11 patient.

12 What is lacking in the art is a rapid immunoassay,
13 sensitive in serum, to detect levels of markers involved in
14 autoimmune diseases, specifically MS, wherein results are
15 definitive and the requirements of the patient are simple. It
16 is the intention of the present invention to offer an assay
17 format which can be used as a rapid manual test to be
18 administered at the point-of-care at any location.

19
20 SUMMARY OF THE INVENTION

21 The present invention relates to the detection of
22 biomolecules linked to multiple sclerosis, along with methods
23 to assess severity of disease with routine monitoring. The
24 presence, in excess, of autoantibodies to myelin basic protein

1 in blood has now been shown to be indicative of MS.

2 The present invention contemplates assembly of a kit for
3 diagnosis of MS and monitoring of previously diagnosed MS
4 patients, thus providing simple and definitive results, on a
5 regular basis, which may assist in predicting an upcoming acute
6 attack. Consequently, preventive therapy could be properly
7 implemented to alleviate the recurring, damaging attacks MS
8 patients currently face without warning.

9 At the present time, there is no simple diagnostic test
10 for MS. The diagnosis of MS currently remains primarily one of
11 clinical evaluation. Since the time of Charcot's first
12 observations, the definitive aspect of the diagnosis of MS has
13 centered around the demonstration of lesions disseminated in
14 time and space. In order to justify this diagnosis, a patient
15 must either present with the occurrence of more than one attack
16 (exacerbation/relapse) or progression of symptoms over many
17 months, as well as, evidence of multiple discrete anatomical
18 loci of disease in the white matter of the central nervous
19 system (CNS) (Miller, 1998). Perhaps the most widely accepted
20 diagnostic scheme was put forth by Schumacher and his
21 colleagues in 1965. Patients are classified as having
22 "clinically definite, probable, or possible" MS depending on
23 the number of the following criteria described below which are
24 applicable (Miller, 1998):

1. Age at onset between 10-50 years;
2. Objective neurological deficits present on examination referable to CNS dysfunction;
3. Neurological symptoms and signs indicative of CNS white matter disease;
4. Dissemination in time: two or more attacks (lasting at least 24 hours) and separated by at least one month (an attack is defined as the appearance of new symptoms or signs or worsening of previous ones) or the progression of symptoms and signs for at least six months;
5. Dissemination in space: two or more noncontiguous anatomical areas of brain involved;
6. No alternative clinical explanation.

The diagnosis of "clinically definite MS" requires that patients meet five or six of the criteria, always including the last one. Patients who fulfill fewer than five of the criteria, but always including the last, are diagnosed with either "clinically probable MS" or "clinically possible MS".

For the purposes of demonstrating this invention, the bodily fluids collected and analyzed will include, but are not limited to, blood and blood products. Through a battery of optimization experiments, an enzyme-linked immunosorbent assay (ELISA) to measure MBP autoantibodies in the blood of MS patients and those not yet diagnosed has been developed.

1 Precision studies have shown that the assay is precise and
2 repeatable at both high and low antibody titers. Analytic
3 recovery experiments indicate that normal plasma constituents
4 do not interfere with the accurate detection of MBP
5 autoantibodies using this ELISA and that both serum and plasma
6 are suitable matrices for their measurement. Hemolyzed,
7 icteric, and lipemic blood samples do not interfere in the
8 assay and circulating concentrations of several common MS drugs
9 do not inhibit the detection of MBP autoantibodies. The
10 developed assay is simple, inexpensive, and rapid (2 hours).

11 More specifically, the method of the subject invention
12 involves an ELISA which comprises: (1) mixing a sample of body
13 fluid, particularly blood or a blood product, from a mammal,
14 usually a human, with at least one compound effective to
15 optimize the signal to noise ratio (the compound exemplified
16 herein is heparin); (2) contacting the sample with an
17 immunosorbent coated with at least one protein associated with
18 MS (exemplified herein is myelin basic protein) having a high
19 specific activity for at least one autoantibody (anti-MBP IgG
20 or IgM); and, (3) determining an amount of at least one
21 autoantibody bound by the protein or proteins on the
22 immunosorbent using an antibody composition having an affinity
23 for the autoantibody, illustrated by, but not limited to,
24 purified anti-human IgG/IgM conjugated to horseradish

1 peroxidase (HRP) that is operably linked to a signal generating
2 system, illustrated by, but not limited to,
3 tetramethylbenzidine (TMB) substrate.

4 Accordingly, it is an objective of the instant invention
5 to provide a clinically sensitive and reliable immunoassay to
6 measure autoantibodies, specifically to MBP, in the blood of MS
7 patients.

8 It is a further objective of the instant invention to
9 provide a less invasive and less costly alternative to current
10 techniques.

11 It is a further objective of the instant invention to
12 provide a rapid method of detection of MS by an immunoassay.

13 It is yet another objective of the instant invention to
14 provide a simple, definitive test for diagnosis of MS.

15 It is a still further objective of the invention to supply
16 a means of determining disease sub-classification and relative
17 disease activity as determined by routine patient monitoring.

18 It is yet another objective of the instant invention to
19 provide a diagnostic assay device for use in the method.

20 Other objects and advantages of this invention will become
21 apparent from the following description taken in conjunction
22 with the accompanying drawings wherein are set forth, by way of
23 illustration and example, certain embodiments of this
24 invention. The drawings constitute a part of this

specification and include exemplary embodiments of the present invention and illustrate various objects and features thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the potential mechanisms for demyelination within the blood-brain barrier;

Figure 2 shows the MBP autoantibody IgM levels in normal vs. clinically definite MS patients, wherein levels in MS patients are generally elevated (+2SD above normal levels);

Figure 3 shows the results of myelin basic protein (MBP) autoantibody IgG levels in clinically stratified MS stable vs. relapse patients, wherein relapse patients exhibit more elevated levels;

Figure 4 demonstrates the types of disease course in multiple sclerosis (MS), which are relapse-remitting, secondary-progressive, and primary-progressive;

Figure 5 illustrates the ELISA protocol;

Figure 6 illustrates the limited sensitivity of a Western blot;

Figure 7 shows the results of MBP autoantibody IgG levels in normal vs. clinically definite MS patients, wherein levels in MS patients are generally elevated (+2SD above normal levels);

Figure 8 demonstrates Receiver Operating Curves (ROC);

Figure 9 demonstrates the diagnostic value of the developed ELISA;

1 Figure 10 demonstrates the added sensitivity when markers are
2 utilized in combination.

3
4 DETAILED DESCRIPTION OF THE INVENTION

5 The purpose of this study was to develop an immunoassay to
6 measure MBP autoantibodies in the blood of MS patients, thereby
7 avoiding the invasive technique of lumbar puncture required for
8 CSF collection. By examining matrix effects within the
9 developed assay, both serum and plasma samples may be used. In
10 addition, different autoantibody classes, that is IgG and IgM,
11 may better indicate the disease status of the individual
12 patient. However, other body fluids may apply which require
13 minimally invasive techniques to obtain, such as saliva, and
14 are contemplated by the present invention.

15 Given the invasive nature of lumbar puncture, its
16 unsuitability as a widely accepted diagnostic measure and since
17 the majority of these other markers have been measured in the
18 CSF, clinical sensitivity and specificity of MBP autoantibodies
19 and other markers must be re-confirmed in the blood of MS
20 patients. The diverse clinical manifestations of MS,
21 variations in immunological evidence of pathology between
22 patients, and the presence of immune responses to different
23 autoantigens suggests not only that MS is a heterogeneous
24 disease but also emphasizes that accurate diagnosis with high

1 sensitivity and specificity may mandate that multiple markers
2 be incorporated into one test for the definitive diagnosis and
3 progressive monitoring of the disease.

4 Due to the highly cationic nature of MBP, its charge may
5 be the contributing factor for high background in a common
6 enzyme-linked immunosorbent assay (ELISA). Non-specific charge
7 interactions typically plague the sensitivities of ELISAs using
8 positively charged proteins (Pesce et al., 1986). This
9 obstacle was recognized early during the development of the
10 disclosed ELISA protocol.

11 In the determination of the assay's clinical utility,
12 sera/plasma of clinically definite MS patients should exhibit
13 elevated titers of MBP autoantibodies when compared with titers
14 in healthy controls, therefore offering a high clinical
15 sensitivity and specificity. If MBP autoantibodies are
16 involved in the pathogenesis of demyelinating lesions (Figure
17 1), we expect that their levels should increase during periods
18 of active demyelination, resulting in relapse or exacerbation
19 of clinical signs in MS patients. Referring to Figure 1, which
20 is an excerpt from the Doctoral Thesis of Baldwin Mak,
21 University of Toronto, 1999, demyelination begins with the
22 disruption of the bilayer 1. Deimination of MBP by
23 peptidylarginine deiminase (PAD) 2 and further disorganization
24 of the bilayer 3 then occurs. This results in the shedding of

1 MBP 4 and subsequent activation of astrocytes and induction of
2 astrogliosis and homing to myelin 5. As the pathway continues,
3 digestion of MBP by cathepsin D and matrix metalloproteinase
4 (MMP) generates MBP peptides 6. Lymphocytes become sensitized
5 7 and MMP digests the BBB, thus allowing sensitized lymphocytes
6 to cross the barrier 8. The autoimmune phase has commenced 9.
7 The diagnostic utility of the developed MBP autoantibody assay
8 will be compared with the clinical utility of assays measuring
9 other biochemical markers for MS.

10 Measurement of plasma MBP autoantibodies (IgG) by enzyme-
11 linked immunosorbent assay (ELISA) is effective in achieving
12 the clinical objectives of high sensitivity (77%) and
13 specificity (95%). Circulating anti-MBP IgG in MS appears to
14 be an indicator of disease activity as levels were increased in
15 patients who were experiencing relapse. The measurement of MBP
16 autoantibodies by ELISA was also shown to be a more sensitive
17 technique than Western blotting and appears to have the highest
18 opportunity for clinical relevance compared with the
19 measurement of autoantibodies to PLP, NSE, and S-100B alone, or
20 presence of the proteins themselves.

21 With respect to anti-MBP IgM levels, only 30% of the MS
22 patients exhibited circulating elevated titers, as measured by
23 ELISA (Figure 2). Although one patient with an extremely
24 elevated anti-MBP IgM level was experiencing a first relapse in

1 MS, no significant difference was found between relapsing and
2 stable patients on the whole. In fact, no definitive feature
3 of the patients with elevated anti-MBP IgM could be
4 ascertained. Elevated IgM in the CSF during episodes of
5 relapse has been previously established in the literature,
6 however, little focus has been devoted to determining the
7 antigenic characterization of this IgM response. In one group
8 of RRMS patients, MBP-specific IgM responses correlated well
9 with CSF IgM indices (Annuziata et al., 1997). Furthermore,
10 patients with high IgM anti-MBP suffered from fewer attacks,
11 with a less rapid frequency and exhibited a decrease in EDSS
12 score more readily as compared with low IgM anti-MBP patients.
13 It appears that anti-MBP IgM primary response is the product of
14 specific antigenic stimulation. Patients with high IgM
15 followed a more favorable/benign course during follow-up.
16 Therefore, long-term monitoring of this type of patient
17 including duration and time between attacks, may be useful in
18 the determination of the significance of elevated anti-MBP IgM
19 in the circulation.

20 Levels of IgG in the stratified patient cohort were
21 significantly elevated in the relapsing patients versus
22 patients in a stable/remitting phase of their disease (Figure
23 3). Thus, it appears that circulating anti-MBP IgG is
24 indicative of disease activity. This observation was also

1 reported by numerous investigators who measured anti-MBP IgG in
2 the CSF. The nature of the disease dictates that relapses have
3 various frequencies of occurrence, ranging from a couple months
4 to several years. Therefore, MBP autoantibodies may be
5 reliably detected in the circulation of MS patients. Moreover,
6 the IgG class of antibodies may be a better indicator of
7 disease activity than IgM. However, IgM may prove useful as an
8 indicator of an initial episode or in predicting disease
9 progression.

10 In order to further evaluate the specificity of the
11 autoantibody response to MBP in MS patients, other autoantibody
12 responses to both myelin proteins, as well as, non-myelin CNS-
13 specific proteins were examined. Since MBP autoantibodies were
14 the most elevated in the MS patients tested, as compared with
15 PLP, NSE, or S-100B autoantibodies, the autoantibody response
16 in MS appears to be specific for myelin basic protein. The 6%
17 of patients who presented elevated anti-PLP IgG all exhibited
18 elevated titers of MBP autoantibodies as well.

19 Analysis of autoantibodies for all three proteins (PLP,
20 NSE, and S-100B) by Student's t-test revealed that there was no
21 significant difference in the levels between normal and MS
22 patients. ELISA was performed for proteolipid protein (PLP),
23 which accounts for 50% of the total myelin protein, neuron
24 specific enolase (NSE), a neuronal marker, and S-100B, an

astrocyte marker.

In general, patients with autoimmune diseases tend to be reactive to multiple antigens. In order to accurately validate the diagnostic potential of the MBP autoantibody assay, it is critical to demonstrate that the antibody response to MBP was specific for MS patients. Although the autoantibody response in MS appears to be specific for myelin basic protein, the level of autoantibodies specific for NSE may prove beneficial as an indicator for axonal degeneration secondary to demyelination and the level of S100B may indicate the extent of gliosis or astrocyte/oligodendrocyte breakdown in certain MS patients.

It became apparent that the addition of a polyanion was required to aid in the charge neutralization of the MBP. After optimization experiments were performed, heparin was chosen because it significantly improved the distinction between control and MS patients while maintaining an excellent signal to noise ratio with the positive control. The sensitivity of the ELISA largely depends on the reduction of non-specific binding by other serum factors.

As a heterogeneous disease, clinical presentation and neuropathology are diverse between patients (Laman et al, 1998). Recent recommendations for the development of novel diagnostic measures in MS incorporate the fact that MS is a

1 disease mediated immunologically; markers may be identified
2 more easily in accessible peripheral fluids, i.e. blood, urine
3 and CSF (Laman et al., 1998). Such a marker should meet
4 acceptable assay performance requirements and correlate with
5 disease stage.

6 Based on the evidence, should MBP autoantibodies in the
7 blood of MS patients mimic the diagnostic benefit of MRI and
8 identify possible MS patients as experiencing disease, whether
9 used alone or in conjunction with MBP or Tm concentrations, MBP
10 autoantibody measurement would offer clinicians a rapid,
11 sensitive, relatively non-invasive, and inexpensive tool for
12 the diagnosis and monitoring of the progression of MS.

13 The overall efficiency of a fully developed immunoassay is
14 determined by the proportion of all patients for whom the test
15 correctly predicts the presence or absence of disease (Micallef
16 and Ahsan, 1994). The clinical utility of MBP autoantibody
17 measurement was examined by using the developed ELISA to
18 measure anti-MBP IgG and IgM in clinically definite MS
19 patients. The present invention is based on the hypothesis
20 that MBP IgG should correlate with disease activity. In
21 addition, detection by Western blot, which has been employed by
22 other investigators, of anti-MBP IgG in the sera of MS patients
23 should demonstrate less sensitivity in comparison. Moreover,
24 if the autoimmune response in MS is specific to MBP,

1 autoantibodies to other myelin and non-myelin proteins should
2 be negligible in comparison.

3 By "sample", what is meant is a volume of body fluid
4 (preferably obtained in a non-invasive manner), such as blood
5 or blood products, saliva, or any body fluid from which a
6 meaningful analysis may be performed, which is obtained at one
7 point in time. Further, all the markers can be measured with
8 one assay device or by using a separate assay device for each
9 marker in which case aliquots of the same fluid sample can be
10 used or different fluid samples can be used. It is apparent
11 that the analyses should be carried out within some short time
12 frame after the sample is taken, e.g., within about one-half
13 hour, so the data can be used to prescribe treatment as quickly
14 as possible. It is preferred to measure each of the markers in
15 the same single sample, irrespective of whether the analyses
16 are carried out in a single analytical device or in separate
17 such devices so the level of each marker is measured
18 simultaneously and the resulting presence in a single sample
19 can be used to provide meaningful data.

20 As used herein the term "marker", "biomolecule",
21 "biochemical marker", or "marker protein", refers to any
22 enzyme, DNA, RNA, carbohydrate, steroid, lipid, protein,
23 polypeptide, peptide, isomeric form thereof, immunologically
24 detectable fragments thereof, or other molecule that is

1 released from the brain during the course of MS pathogenesis.
2 Such markers include, but are not limited to, any unique
3 proteins or isoforms thereof that are particularly associated
4 with the brain and/or proteins or isoforms thereof that are
5 found in tissues other than the brain.

6 By "immunologically detectable" is meant that a marker or
7 protein or fragments thereof contain an epitope which is
8 specifically recognized by a cognate antibody or antibody
9 reagents used in the assay.

10 The term "diagnostic" is meant that a marker or protein or
11 fragments thereof is present at statistically significant
12 levels indicative of initiation of disease state or state of
13 disease activity, e.g. relapsing or progressing of disease
14 activity.

15 The term "monitor" is used herein to determine the
16 occurrence, to distinguish type, to measure severity, or to
17 conclusively track progression of disease. In addition, the
18 present invention relates to the usefulness of continued
19 monitoring of MS patients for a period of time. This type of
20 assessment could be very useful in the proper treatment of
21 persons suffering from MS.

22 The terms "above normal" and "upregulated" are used herein
23 to refer to a level of a marker that is greater than the level
24 of the marker observed in normal individuals, that is,

1 individuals who are not undergoing disease activity related to
2 MS. For some markers, no or infinitesimally low levels of the
3 marker may be present normally in an individual's body fluid,
4 such as blood. For other markers analyzed, according to the
5 invention, detectable levels may be present normally in a body
6 fluid. Thus, these terms contemplate a level that is
7 statistically significant or significantly above the normal
8 level found in individuals.

9 The term "statistically significant" or "significantly"
10 refers to statistical significance and generally means a two
11 standard deviation (2SD) above normal, or higher, concentration
12 of the marker. The assay method by which the analysis for any
13 marker protein is carried out must be sensitive to be able to
14 detect the level of the marker which is present over the
15 concentration range of interest and also must be highly
16 specific.

17 One embodiment is a method of diagnosing or monitoring MS
18 in a mammal, preferably a human. A sample body fluid is
19 obtained from the mammal, wherein in a preferred embodiment the
20 body fluid is blood or blood products, e.g. serum, plasma and
21 the like. The sample is contacted with at least one protein
22 associated with multiple sclerosis, by way of an ELISA, and a
23 level of at least one autoantibody specific for at least one
24 protein in the sample is determined. The levels are compared

1 with the level of the at least one autoantibody with
2 statistically significant levels thereof, wherein diagnosis or
3 monitoring of MS is achieved. In a preferred embodiment, the
4 protein is MBP and the autoantibody is anti-MBP IgG, anti-MBP
5 IgM, or both.

6 In a further embodiment, a kit may be assembled for
7 diagnosing MS or monitoring disease state in MS patients, which
8 comprises at least one biomolecule or an immunologically
9 detectable fragment thereof which is upregulated in MS patients
10 and being immobilizable on a solid support, where the
11 biomolecule or biomolecules has an affinity for at least one
12 additional biomolecule whose presence is diagnostic of MS. At
13 least one labeled biomolecule having a binding affinity for the
14 at least one additional biomolecule would be included; whereby
15 the performance of at least one analysis determinative of the
16 presence of statistically significant levels of the biomolecule
17 or biomolecules or an immunologically detectable fragment
18 thereof, is carried out on a sample of body fluid and provides
19 a means for diagnosing or monitoring disease state.
20 Preferably, a biomolecule would be represented by MBP whereby
21 an additional biomolecule would be anti-MBP IgG, anti-MBP IgM,
22 or both. The labeled biomolecule would preferably be anti-
23 human IgG/IgM (depending on the choice of additional
24 biomolecule) conjugated to HRP.

1 All samples would be taken at the same time or at
2 different time periods with monitoring of disease activity
3 generally beneficial with one or more samples obtained at
4 various times during disease course.

5 Disclosed herein is a unique ELISA procedure for detection
6 of MS-specific autoantibodies. The following examples are
7 given for the purpose of illustrating various embodiments of
8 the invention and are not meant to limit the present invention
9 in any fashion.

10 EXAMPLE 1

11 Materials and Methods

12 MS patient sample size was determined by currently
13 available patients. Under the assumption of a conservative
14 estimate of a two standard deviation (2SD) difference between
15 MS patients and controls, the minimum sample size of 10
16 patients provides 80% power to detect a type I error at $p=0.01$.

17 Consecutive MS patients meeting the diagnosis of
18 clinically definite MS, as described by Poser et al. (1983),
19 seen in consult at the St. Michael's Hospital (SMH), MS Clinic,
20 Toronto, Ontario, were offered study inclusion. Baseline
21 assessments were made in the clinic including date of birth
22 (DOB), sex, date of blood collection, when available: date of
23 symptom onset and date of diagnosis, as well as a detailed
24 neurologic examination, determination of disease severity score

1 (EDSS; Expanded Disability Scaled Score) and categorization of
2 current disease status (Figure 4) (active relapse, remission,
3 primary-progressive, secondary-progressive).

4 Blood samples from ninety eight apparently healthy
5 individuals, with no previous symptoms of MS, were drawn at Syn
6 X Pharma, Inc. DOB and gender were recorded at the time of
7 blood collection and a general health questionnaire was used to
8 record any other diseases in the individual donors.

9 Following patient consent, phlebotomy was performed on
10 ninety-six MS patients. Each patient was assigned an SMH
11 number at the time of blood collection to ensure the
12 confidentiality of the patients and provide anonymous analysis
13 of the blood until clinical utility of the test was assessed.
14 The blood was collected into matched heparinized and serum
15 Vacutainer tubes (Beckton-Dickenson) and centrifuged at 3,000
16 rpm for 30 minutes. Each patient's plasma and serum fractions
17 were aliquoted and frozen at -20°C until use. The patient
18 information was obtained from the clinic charts after analysis
19 by ELISA.

20 EXAMPLE 2

21 MBP Autoantibody ELISA

22 Microtiter Plate Preparation

23 All odd column wells of 96 well microtiter plates
24 (MaxiSorp, Nunc) were coated with 125 μ L of 8 mg/L

unfractionated myelin basic protein (bovine) in 100 mM carbonate/bicarbonate buffer, pH 9.6. All even column wells were coated with 125 mL of 100 mM carbonate/bicarbonate buffer (modified Crimando and Hoffman, 1992). Plates were sealed with ELISA plate sealer (Costar) and incubated at 4°C overnight. Plates were then washed three times with 10 mM PBS + 0.05% Tween-20, 300 mL/well. All wells were subsequently blocked with 250 mL/well of 2% w/v BSA (Equitech-Bio) in carbonate/bicarbonate buffer, to reduce non-specific binding, and were incubated at 4°C overnight.

ELISA Protocol (Figure 5)

Plates were washed three times with 10 mM PBS + 0.05% Tween-20. Plasma test samples were diluted in the dilution buffer which consisted of 10 mM PBS, 0.05% Tween-20 and 5 USP/mL heparin (Sigma) (Pesce et al., 1986) at a working dilution of 1:320. Added to the matched MBP and adjacent MBP-free wells was 100 µL of each diluted test sample. Affinity purified goat polyclonal antibody against bovine unfractionated MBP was diluted in a calibrant of normal human plasma, diluted at 1:320 in the dilution buffer, and tested at 0, 25, 50, 100 and 200 ng/mL to form a standard curve. Negative controls were run in quadruplicate and consisted of neat dilution buffer. The wells were incubated for 1 hour at room temperature. Plates were then washed three times with 10 mM PBS + 0.05%

1 Tween-20. Wells containing test samples and the negative
2 control were incubated with 100 μ L/well of goat anti-human IgG
3 (Fc) conjugated to horseradish peroxidase (Jackson) or goat
4 anti-human IgM conjugated to horseradish peroxidase (Jackson),
5 diluted 1:15,000 or 1:20,000 respectively, in dilution buffer
6 for 1 hour at room temperature. The wells containing the goat
7 polyclonal antibody were incubated with 100 μ L of donkey anti-
8 goat IgG conjugated to horseradish peroxidase (Jackson),
9 diluted 1:7,000 in dilution buffer for 1 hour at room
10 temperature. After three washes with 10 mM PBS + 0.05% Tween-
11 20, 100 μ L of tetramethylbenzidine (TMB) (Moss) was added to
12 each well and incubated in the dark for 2.5 minutes. The
13 reaction was stopped with 1 N H_2SO_4 , 100 μ L/well. Optical
14 density was read on a SoftMax microtiter plate reader
15 (Molecular Devices) at 450nm. Absorbance values from the non-
16 coated well were subtracted from the OD 450nm value in the MBP
17 coated well from the same sample. This subtracted value (S-
18 value) reflects the specific antibodies present in the sample.
19 The subtracted values obtained from the standard curve have
20 been used to quantitate the amount of IgG in each test sample.

21 EXAMPLE 3

22 Western Blot Protocol for the Detection of MBP Autoantibodies 23 IgG

24 The equivalent of 2 μ g unfractionated myelin basic protein

1 (bovine) per lane was separated by 12.5% sodium dodecyl sulfate
2 polyacrylamide gel electrophoresis (SDS-PAGE) at 180 volts(V)
3 for 60 minutes at room temperature (Laemmli, 1970) as well as,
4 6 mL of precision molecular weight markers (BIORAD). The gels
5 were then transferred to Immobilon P PVDF membranes (Millipore)
6 at 100 V for 180 minutes at 4°C using 10% methanol in transfer
7 buffer. Following electroblotting, the membranes were blocked
8 with 5% Blotto/TBS and allowed to incubate for 60 minutes,
9 shaking at room temperature and then incubated at 4°C
10 overnight. The membranes were washed for 30 minutes at room
11 temperature, shaking with Tris buffered saline + Tween-20
12 (TTBS). The membranes were secured in the MiniCell Protean III
13 multi-sample apparatus. Thirty-eight normal serum samples and
14 forty-two clinically definite MS samples were randomly chosen
15 and diluted 1:10 in 1% Blotto/TBS. Diluted serum samples (240
16 mL of each sample) were loaded into separate lanes on the
17 apparatus. The diluted serum samples were incubated for 60
18 minutes, shaking at room temperature and all sera was
19 subsequently aspirated off the membrane using a vacuum pump.
20 The membranes were washed three times, each for 5 minutes with
21 TTBS. Goat anti-human IgG (Fc) conjugated to horseradish
22 peroxidase (Jackson) was diluted 1:2,000 in 1% Blotto/TTBS.
23 Membranes were incubated with the conjugated antibody for 120
24 minutes, shaking at room temperature. The membranes were

1 subsequently washed three times, each for 5 minutes, shaking at
2 room temperature with TTBS. The immunoblots were developed
3 using a TMB substrate kit as per the manufacturer's
4 instructions for 3 minutes. The reaction was stopped with
5 ultra-filtered water.

6 In order to evaluate Western blot analysis as a secondary
7 method of MBP autoantibody detection, normal and clinically
8 definite MS patient samples were analyzed for MBP specific IgG
9 (Figure 6). Sensitivity measurement at low titers of MBP
10 autoantibodies in the circulation and confirmation of
11 sensitivity regarding an ELISA protocol of the instant
12 invention were focused upon. None of the MS patient samples
13 demonstrated a definitive band at 18.5 kDa, corresponding to
14 MBP. This may be explained by the fact that the variation in
15 background for the MS patients tested by Western blot can not
16 be adjusted for as is done in the ELISA format, by the addition
17 of heparin (Alcantara et al., 1999) and the subtraction of an
18 individual sample background. This merely compounds the
19 difficulty in achieving reasonable sensitivity by this method.

20 EXAMPLE 4

21 MBP Autoantibody ELISA Development And Assay Performance

22 Numerous optimization experiments must be conducted during
23 the development of an immunoassay in order to maximize analytic
24 and clinical sensitivity, as well as minimize non-specific

1 interference within the assay. Since most assays for specific
2 antibodies are qualitative, these experiments generate an assay
3 which minimizes false positive results and allow for a low
4 detection limit required to detect low titer antibody levels
5 (Micallef and Ahsan, 1994), while maintaining low levels of
6 imprecision. Upon the completion of the fully optimized assay,
7 experiments to determine performance characteristics are
8 conducted to show that the assay is precise and repeatable.
9 For example, MaxiSorp C plates offered a more reliable
10 measurement of antibody while maintaining an excellent signal
11 to noise ratio. Therefore, MaxiSorp microtiter plates were the
12 optimal solid phase for this assay.

13 EXAMPLE 5

14 Dilution Factor Determination

15 The optimal serum/plasma dilution factor was chosen by
16 running eleven normal samples and five clinical samples in the
17 anti-MBP IgG ELISA. The choice of 1:320 as the plasma dilution
18 was based on the fact that the point of inflection for the
19 majority of the normal samples in their serial dilution was
20 observed at this dilution factor.

21 As previously mentioned MBP is a cationic protein and
22 therefore non-specific charge interactions were hypothesized to
23 contribute to the lack of distinction between normal and MS
24 patient MBP autoantibody titers. Heparin (5 USP) was placed in

1 the plasma dilution buffer due to the success with the
2 polyclonal control (rabbit). At the optimal dilution factor of
3 1:320, only 2 of the initial 24 MS patient samples exhibit
4 antibody titers elevated above the mean +2SD of the normal
5 range (n=11). Clinical detection, via sensitivity,
6 dramatically improved when the antibody dilution buffer was
7 modified to include heparin. This affords a possible reason as
8 to why some investigators achieved successful detection of MBP
9 autoantibodies where others had previously failed. Upon
10 examination of those reports denying the presence of anti-MBP,
11 the assay protocols employed simple buffers, which did not
12 appear to be specific for such a cationic protein.

13 Plasma from two separate MS patient samples was added to
14 plasma from each of three normal donors and were assayed for
15 anti-MBP IgG. The S-value for both MS patient samples was
16 within a 5% margin of recovery. Traditionally, analytic
17 recovery experiments are performed by adding a known
18 concentration of purified analyte (in this case purified anti-
19 MBP IgG) into normal samples. However, this technique can not
20 be performed for this assay since purified MBP IgG is not
21 commercially available. Nevertheless, this recovery experiment
22 illustrates that MBP autoantibody titers are not subject to
23 interference by normal plasma constituents.

24 The effect of interfering substances such as hemoglobin,

lipids, or bile acids are generally appreciated by the inclusion of hemolyzed, lipemic, or icteric blood in the normal cohort of samples examined (Miller and Levinson, 1996). However, it is important to test these possible interfering substances in the developed assay by adding known quantities of hemoglobin, lipid, and bilirubin into patient samples. Moreover, the extent to which specific pharmacotherapeutic drugs for the disease of interest interfere with the testing of the analyte is also critical. By evaluating patient samples containing added increasing quantities of common therapeutics, the extent of possible drug interference can be determined.

In order to assess whether standard interference compounds, such as hemoglobin, bilirubin, and lipid prevent the accurate measurement of MBP autoantibodies, three concentrations each of hemoglobin, bilirubin, and triglyceride were added to the plasma from one MS patient. Each sample was assayed for MBP IgG in duplicate. When compared with the subtracted OD values (S-values) achieved by the control samples (MS patient plasma without any additional compounds), samples with elevated hemoglobin, bilirubin, and triglyceride yielded similar OD values. The three concentrations of each compound tested, represent values outside the normal range, as well as within the normal range. Therefore, MS patients who present with hemolyzed, icteric, or lipemic blood samples can still be

1 reliably tested for MBP autoantibodies by this assay.

2 Patients with autoimmune disease generally attempt to
3 control their symptoms with some form of agent. Many patients
4 prefer such pharmaceutical agents as methylprednisolone (SOLU-
5 MEDROL), IFN β -1a (REBIF/AVONEX), and glatiramer acetate
6 (COPAXONE). Interferon β (IFN β)-1b (BETASERON), and IFN β -1a
7 (REBIF/AVONEX) are both considered effective "first line"
8 treatments in RRMS, in that they reduce relapses and MRI
9 lesions, both indicators of disease activity in RRMS.

10 Although the mechanism of action has not been clearly
11 defined for the β -interferons, their effects are likely that of
12 immunomodulation because β -interferons are anti-inflammatory
13 cytokines. They may also have significant capability of
14 slowing down lymphocytic trafficking across the BBB, a theory
15 suggested because they tend to suppress the development of
16 gadolinium (Gd)-enhancing MRI lesions.

17 Glatiramer acetate (COPAXONE) is a linear polymer of the
18 L-amino acids: glutamate, tyrosine, alanine, and lysine, which
19 comprise an immunodominant region of myelin basic protein.
20 COPAXONE'S efficacy lies in its ability to reduce the number of
21 relapses in the individual patient. COPAXONE is thought to
22 downregulate immune responses directed against myelin
23 components by stimulating the generation of suppressor cells
24 that are capable of reducing immune response by release of

1 anti-inflammatory cytokines. Although this drug has been shown
2 to reduce relapse rate and MRI lesion activity, COPAXONE has no
3 effect on short-term disease progression as measured through
4 the EDSS.

5 Methylprednisolone (SOLU-MEDROL) is a corticosteroid which
6 is used to treat inflammation. SOLU-MEDROL is commonly
7 administered to MS patients during acute relapse because it is
8 deemed to have the effect of closing the damaged BBB and
9 reducing inflammation in the CNS (van den Noort and Holland,
10 1999).

11 Paclitaxel (TAXOL) is a well known anti-cancer agent.
12 Recent evidence (Cao et al., 2000) has shown that paclitaxel is
13 an effective agent in attenuating the clinical manifestations
14 of experimental allergic encephalomyelitis (EAE), the classic
15 T-cell-mediated animal model of MS. TAXOL, as a valuable early
16 treatment for MS, is contemplated by this invention.

17 In the assessment of possible interference of common MS
18 pharmaceutical agents, increasing doses of SOLU-MEDROL,
19 REBIF/AVONEX, COPAXONE and TAXOL were added to the diluted
20 plasma of one MS patient. With the exception of SOLU-MEDROL,
21 the remaining three drugs did not significantly decrease the
22 endogenously detected titer of MBP autoantibodies. SOLU-MEDROL
23 concentrations commencing with 375 mg/mL significantly
24 inhibited the accurate detection of MBP autoantibodies in the

1 patient's sample. The recommended dose for this drug ranges
2 between 10 - 6,000 mg. Since 375 mg/mL translates to over 50
3 g in the average individual, the detection of MBP IgG in a
4 patient receiving this treatment would remain unaffected. MBP
5 IgG may be a useful biochemical marker for monitoring MS
6 progression in patients undergoing novel therapies.

7 In order to quantitate the results achieved in this assay,
8 a standard curve using affinity purified anti-MBP IgG, raised
9 in goat, was obtained. Using three separate assay runs, the
10 standard curve exhibits linearity from 0 to 100 U/mL. This
11 standard curve has been used to quantitate the amount of IgG in
12 each patient sample.

13 EXAMPLE 6

14 MBP Autoantibody ELISA

15 Seventy-four MS patients were stratified on the basis of
16 disease status at the time of blood collection, irrespective of
17 disease course. As illustrated in Figure 3, patients in
18 relapse (n=26) had MBP IgG autoantibody levels significantly
19 elevated ($p < 0.001$, Student's t-test) compared with those
20 patients in stable courses of the disease (n=48).

21 In a further experiment, ninety-eight normal healthy
22 subjects (age range 20-66, mean age = 36, approximately equal
23 number of males and females) were run on the ELISA, in
24 duplicate. Plasma samples from ninety-four clinically definite

MS patients (age range 18-63, mean age = 38, male and female) (Figure 7) were also run on the ELISA. Using the mean +2SD of the normal subjects studied as the clinical cut-off (dashed line), 77% of the MS patients tested exhibited elevated levels of MBP autoantibodies (IgG) in plasma ($p < 0.001$), whereas only five normal samples exhibited IgG levels marginally above clinical cut-off. Receiver Operating Curves (ROC) (Figure 8) are constructed by plotting the sensitivity vs. the specificity of the test using multiple measurements as possible clinical decision limits. Determination of the optimal clinical cut-off by ROC plot illustrates that using the mean +2SD of the normal samples as the clinical decision limit offers high sensitivity (77%) and specificity (95%) (Figure 9).

Sensitivity and specificity describe attributes of the test when the actual clinical diagnosis is known (i.e. the proportion of those with MS who actually have a positive test result or the proportion of those who are healthy who will have a negative test result). Since, in clinical practice one does not usually know who has the disease, positive predictive value (PPV) and negative predictive value (NPV) are useful in determining how likely a patient is to have the disease given a positive test result. PPV is the proportion of patients with a positive test result who have the disease. NPV is the proportion of patients with a negative test result who do not

1 have the disease. Although useful, PPV and NPV vary according
2 to the prevalence of the disease (i.e. the proportion of people
3 undergoing the test who actually have the disease).
4 Sensitivity and specificity are usually stable regardless of
5 the prevalence of disease in the population in which the test
6 is conducted while predictive values vary considerably in
7 different populations of patients. Likelihood ratios (LR)
8 permit a calculation of the probability of disease for a
9 specific test result and specific disease prevalence.
10 Likelihood ratios with a value greater than 10 are usually
11 judged to be of high diagnostic value. This assay offers an LR
12 of 14.8 and thus is a reliable diagnostic tool for MS.

13 EXAMPLE 7

14 MBP, S-100B, NSE, and Tm ELISA

15 MBP, S-100B, NSE and Tm concentrations were measured in
16 ninety matched MS patient serum samples, by two-site, in-direct
17 ELISA as per the manufacturer's instructions for the Smart MBP
18 kit, Smart S-100B kit, Smart NSE kit and Smart Tm kit (SynX
19 Pharma, Inc.) respectively. The clinical decision limit was
20 determined by the evaluation of 103 normal healthy donors by
21 the manufacturer and was defined as the mean +2SD of the normal
22 population tested. It should be noted that not all MS patient
23 sample analysis could be included in this work, as some of the
24 serum samples were hemolyzed and posed interference problems in

1 the NSE assay.

2 Serum concentrations of Tm, MBP, and NSE were shown to
3 have clinical utility in MS. However, when MBP, Tm, and MBP
4 autoantibody measurements are all considered in the diagnostic
5 work-up of the MS patients, clinical sensitivity increases to
6 95% which can not be achieved by the measurement of any of
7 these markers alone (Figure 10). The adoption of a panel of
8 markers may optimize the usefulness of each of these markers in
9 the diagnosis of MS.

10 All patents and publications mentioned in this
11 specification are indicative of the levels of those skilled in
12 the art to which the invention pertains. All patents and
13 publications are herein incorporated by reference to the same
14 extent as if each individual publication was specifically and
15 individually indicated to be incorporated by reference.

16 It is to be understood that while a certain form of the
17 invention is illustrated, it is not to be limited to the
18 specific form or arrangement herein described and shown. It
19 will be apparent to those skilled in the art that various
20 changes may be made without departing from the scope of the
21 invention and the invention is not to be considered limited to
22 what is shown and described in the specification and
23 drawings/figures.

24 One skilled in the art will readily appreciate that the

1 present invention is well adapted to carry out the objectives
2 and obtain the ends and advantages mentioned, as well as those
3 inherent therein. The embodiments, methods, procedures and
4 techniques described herein are presently representative of the
5 preferred embodiments, are intended to be exemplary and are not
6 intended as limitations on the scope. Changes therein and other
7 uses will occur to those skilled in the art which are
8 encompassed within the spirit of the invention and are defined
9 by the scope of the appended claims. Although the invention
10 has been described in connection with specific preferred
11 embodiments, it should be understood that the invention as
12 claimed should not be unduly limited to such specific
13 embodiments. Indeed, various modifications of the described
14 modes for carrying out the invention which are obvious to those
15 skilled in the art are intended to be within the scope of the
16 following claims.